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A Protein Identical to the Yolk Protein Is Stored in the Testis in Male Red Sea Urchin, *Pseudocentrotus depressus*

TATSUYA UNUMA[1,*], TOHRU SUZUKI[2], TADAHIDE KUROKAWA[2], TAKESHI YAMAMOTO[1], AND TOSHIO AKIYAMA[1]

[1] *Inland Station, National Research Institute of Aquaculture, Tamaki, Mie 519-04, Japan; and* [2] *National Research Institute of Aquaculture, Nansei, Mie 516-01, Japan*

Abstract. Female sea urchins store the major yolk protein (MYP) in ovarian nutritive phagocytes before vitellogenesis. Using immunological procedures, we detected MYP in the testicular nutritive phagocytes of *Pseudocentrotus depressus*, the red sea urchin, and then compared the distribution of MYP between sexes during gametogenesis. MYP was purified from unfertilized eggs by ion exchange chromatography (Q Sepharose) and gel filtration (Superdex 200), and an antiserum (anti-MYP) was raised against MYP. Immunoblot analysis demonstrated that immature testes, as well as ovaries, contained a large quantity of MYP. Immunohistochemistry showed that MYP was distributed in the nutritive phagocytes occupying the follicular lumen in both males and females. In both sexes, as gametogenesis proceeded, the nutritive phagocytes degenerated and the gonadal lumen filled with gametes. MYP accumulated in ripe ova as a yolk protein in the mature ovary. In contrast, MYP was not detected in mature testes, because stored spermatozoa did not react with anti-MYP. We conclude that in male *P. depressus*, MYP is stored in the testicular nutritive phagocytes and utilized as the nutrient source for spermatogenesis.

Introduction

The most abundant yolk protein found in sea urchin eggs is a glycoprotein with a molecular weight of about 180 kDa; this has been termed the major yolk protein or major yolk glycoprotein (MYP; Harrington and Easton, 1982; Kari and Rottmann, 1985; Yokota and Kato, 1988). Unlike other oviparous animals in which vitellogenin, a precursor of MYP, is female specific, sea urchins have an abundant supply of vitellogenin in the coelomic fluid of males as well as females (Harrington and Easton, 1982; Shyu *et al.*, 1986). Shyu *et al.* (1986) found that vitellogenin mRNA is expressed in the intestines and gonads of both sexes in *Strongylocentrotus purpuratus*. This suggests the possibility that in sea urchins vitellogenin performs an unidentified function required by males in addition to its role in vitellogenesis in females.

In female sea urchins, vitellogenin is reported to be incorporated into the nutritive phagocytes (accessory cells) in the previtellogenic ovary for temporary storage, and then transported to the oocytes to be accumulated as MYP (Ozaki *et al.*, 1986; Harrington and Ozaki, 1986). The gonads of male sea urchins also have nutritive phagocytes for nutrient storage (Walker, 1982). However, it is not clear whether the testicular nutritive phagocytes contain vitellogenin.

The main objective of this study is to examine whether male sea urchins store MYP-related proteins in their nutritive phagocytes. To achieve this, we purified MYP from unfertilized eggs of the red sea urchin, *Pseudocentrotus depressus*, and prepared an antiserum against MYP. We compared the distribution of MYP reactivity during gametogenesis in both sexes by immunological analysis.

Materials and Methods

Animals

Six-month-old juveniles of *P. depressus*, hatched and reared at the Fukuoka Prefectural Fish Farming Center,

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* To whom correspondence should be addressed.
Abbreviation: MYP = major yolk protein.

were transferred to the Coastal Station of the National Research Institute of Aquaculture. They were kept in 1000-l tanks supplied with sand-filtered seawater at 30 l · min^{-1}, and reared on kelp, *Eisenia bicyclis*. The animals used for the experiment were 2 or 3 years old.

After the peristomial membrane of the animal was removed, coelomic fluid was collected with Pasteur pipettes. Coelomocytes were removed by centrifugation at 600 × *g* for 5 min. The coelomic fluid was kept at −80°C until use.

Gonads were dissected out and stored at −80°C. Small pieces were fixed in Bouin's solution for histological observations. Paraffin sections of 6-μm thickness were prepared and stained with hematoxylin and eosin. The gonadal maturity of each animal was classified according to the six stages described by Fuji (1960), with some slight modifications as described previously (Unuma *et al.*, 1996).

During the spawning season in November, eggs were obtained by coelomic injection of 20% KCl and then were stored at −80°C.

Purification of MYP

Unfertilized eggs (3 ml) were homogenized with 15 ml of 10 m*M* Tris-HCl buffer containing 10 m*M* NaCl (TBS; pH 8.0) and 0.1 m*M* phenylmethylsulfonylfluoride (PMSF) using Polytron (Kinematica, Switzerland). The homogenate was centrifuged at 25,000 × *g* for 20 min at 4°C, and the supernatant was applied to a HiLoad 16/10 Q Sepharose fast flow column (Pharmacia LKB Biotechnology, Sweden) equilibrated with TBS. After being washed with 40 ml of the same buffer, the retained proteins were eluted with a NaCl linear gradient from 10 m*M* to 1 *M* (200 ml in total) using FPLC (Pharmacia). The flow rate of the column was 2.0 ml · min^{-1}, and 4-ml aliquots of eluate were collected. Fractions rich in MYP were pooled, concentrated threefold using Molcut LGC (Millipore Corp., USA), and applied to a HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated with 10 m*M* Tris-HCl buffer containing 150 m*M* NaCl (pH 8.0). Proteins were eluted with the same buffer at a flow rate of 1 ml · min^{-1}, and fractions of 2 ml were collected. A gel filtration calibration kit (Pharmacia) was used to estimate molecular weight.

Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5% slab gel according to Laemmli (1970). Protein bands were visualized with Coomassie brilliant blue R-250. SDS-PAGE standards (Bio-Rad Laboratories, USA) were electrophoresed for molecular weight calibration. Precast gels (NPU-5L; Atto Corp., Japan) were used for Figures 1 and 5. These gels showed double bands in the low molecular weight end, whereas the gels prepared in our laboratory showed a single band there (Fig. 4). However, Atto Corp. assures us that there is no difference between these gels in comparisons of the middle to high molecular weight ranges.

Preparation of antiserum

The peak fraction from the Superdex 200 gel filtration was electrophoresed on SDS-PAGE under nonreducing conditions. A gel slice containing the 270-kDa band was excised. The protein was eluted from the gel by an Electro Eluter (Bio-Rad), mixed with an equal volume of Freund's complete adjuvant, and then injected subcutaneously into the back of a rabbit once a week. After 4 injections, blood was collected from an ear vein. The blood was centrifuged at 1500 × *g* for 20 min, and the supernatant was collected as antiserum (anti-MYP).

Immunoblotting

Gonads were homogenized with 20-fold TBS and centrifuged at 25,000 × *g* for 20 min at 4°C. Supernatants were used as the gonadal extracts.

To detect the protein reactive with anti-MYP, coelomic fluids and the gonadal extracts were immunoblotted according to Towbin *et al.* (1979). Proteins separated by 5% SDS-PAGE were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was immunostained using anti-MYP and POD Immunostain Set (Wako Pure Chemical Industries, Ltd., Japan).

Immunohistochemistry

To visualize MYP reactivity in the gonads, immunohistochemistry was performed on paraffin-embedded gonads sectioned at 6 μm. After the paraffin was removed, sections were incubated with anti-MYP (1:2000 dilution) and then treated with Histofine SAB-PO Kit (Nichirei, Japan) as described in the manufacturer's instructions. Normal rabbit serum was substituted for anti-MYP to confirm the specificity of the immunohistochemical staining.

Results

Purification of MYP

SDS-PAGE analysis under reducing conditions showed that a 170-kDa protein occurred abundantly in *P. depressus* eggs (Fig. 1a). This protein band was reactive with periodic acid-Schiff (PAS) reagent (data not shown), similar to MYP reported for other sea urchin eggs (Harrington and Easton, 1982; Ozaki *et al.*, 1986; Yokota and Kato, 1988). We concluded that this 170-kDa protein is the MYP of *P. depressus* because of the similarity in

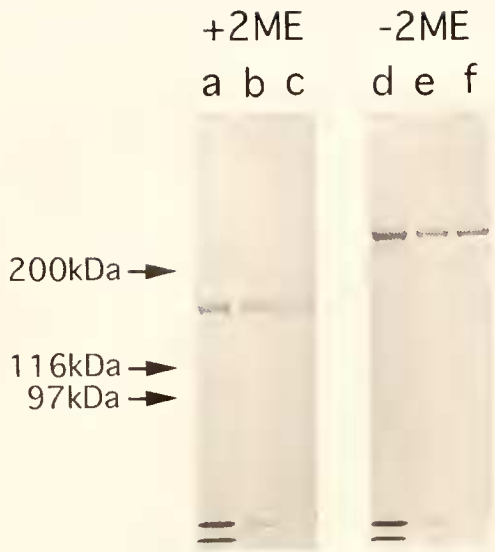

Figure 1. Five percent SDS-PAGE analysis of *Pseudocentrotus depressus* major yolk protein. Lanes a–c are under reducing conditions (+2ME), and d–f are under nonreducing conditions (−2ME). (a, d) Crude extract from unfertilized eggs; total protein = 5 μg. (b, e) Pooled fraction from Q Sepharose (Fig. 2); total protein = 1 μg. (c, f) Pooled fraction from Superdex 200 (Fig. 3); total protein = 1 μg.

molecular weight and the PAS reactivity with other MYP. Under nonreducing conditions, a large quantity of 270-kDa protein was observed instead of the 170-kDa band (Fig. 1d). This suggests that intact MYP consists of subunits.

Egg extract was applied to an ion exchange column using Q Sepharose (Fig. 2). The major protein peak eluted at 280 m*M* NaCl was revealed to be rich in MYP by SDS-PAGE (Fig. 1b, e). After concentration, the fractions were subjected to gel filtration using Superdex 200 (Fig. 3). The major peak eluted at 600 kDa gave a homogeneous 270-kDa band on SDS-PAGE under nonreducing conditions (Fig. 1f). Thus, we concluded that the MYP of *P. depressus* was isolated by a combination of ion exchange chromatography and gel filtration. Under reducing conditions, MYP gave a major 170-kDa band and four minor bands of about 100 kDa (Fig. 1c). These minor bands are probably fragments derived from the intact MYP by proteolysis.

To raise an antiserum against MYP (anti-MYP), we electronically eluted the 270-kDa band from an SDS-PAGE gel run under nonreducing conditions, then immunized a rabbit with the isolated protein (Fig. 1f).

MYP-related proteins in gonads and coelomic fluids

We used SDS-PAGE and immunoblot analyses to test the gonads and coelomic fluids of Stage 1 females and males for the presence of the protein reactive with anti-MYP. A 170-kDa protein was predominant in both female and male gonadal extracts under reducing conditions (Fig. 4b, c). These protein bands were immunoreactive with anti-MYP (Fig. 4l, m). On the basis of the molecular weight and immunoreactivity of the major protein stored in the immature ovary and testis, we concluded that it is identical to MYP. Predominant protein bands with a slightly higher molecular weight (180 kDa) than MYP were detected in the coelomic fluid of both sexes (Fig. 4d, e). In other sea urchins investigated, vitellogenin is the most abundant coelomic fluid protein; it has a molecular weight of about 200 kDa and is immunoreactive with the antiserum against MYP (Harrington and Easton, 1982; Shyu *et al.*, 1986;

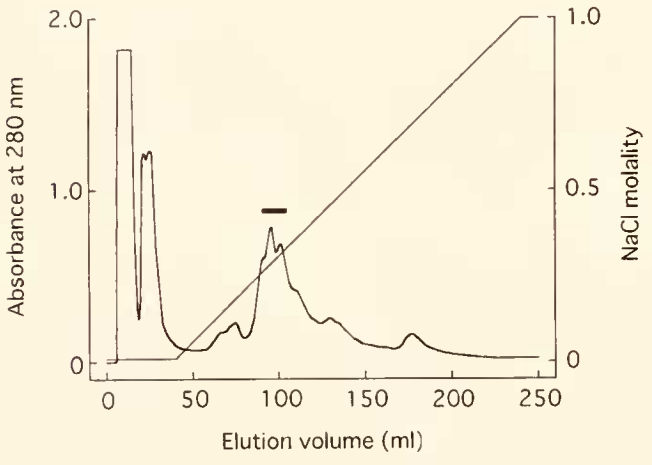

Figure 2. Q Sepharose ion exchange chromatography of extract from unfertilized eggs of *Pseudocentrotus depressus*. Fractions marked with a bar were pooled and concentrated for Superdex 200 gel filtration.

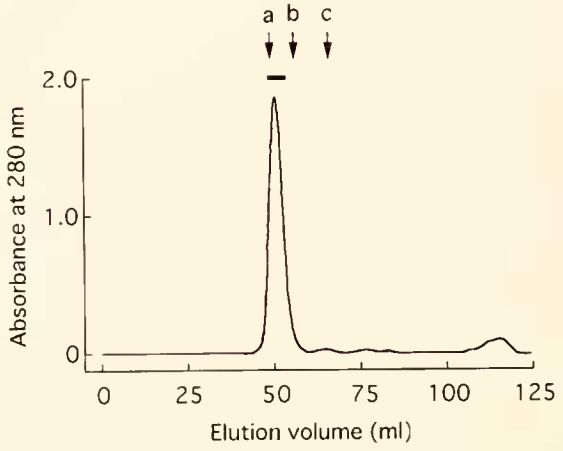

Figure 3. Gel filtration on Superdex 200 of partialy purified major yolk protein. The pooled fraction obtained by Q Sepharose (Fig. 2) was applied to Superdex 200. Fractions marked with a bar were pooled. Thyroglobulin (a; 669 kDa), ferritin (b; 440 kDa), and catalase (c; 232 kDa) were used as standards for molecular weight estimation.

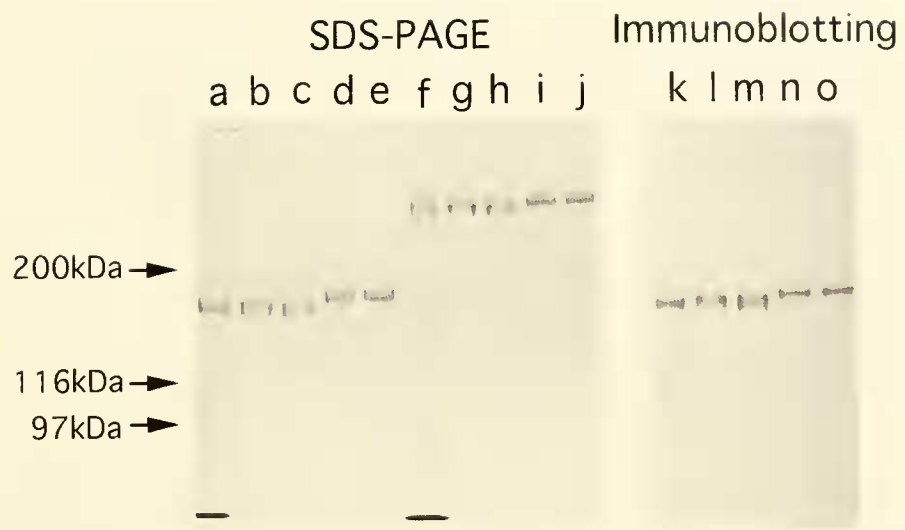

Figure 4. Five percent SDS-PAGE and immunoblotting using anti-MYP of gonadal extracts and coelomic fluids of Stage 1 *Pseudocentrotus depressus*. Lanes a–e and k–o are under reducing conditions, and f–j are under nonreducing conditions. (a, f, k) Egg extract; (b, g, l) ovarian extract; (c, h, m) testicular extract; (d, i, n) female coelomic fluid; (e, j, o) male coelomic fluid. Total protein applied = 10 μg for egg extract, 2 μg for all others.

Cervello *et al.* 1994). The protein of the predominant bands of 180 kDa in the coelomic fluids was identified as vitellogenin on the basis of its reactivity with anti-MYP (Fig. 4n, o) and the similarity of its molecular weight to that of vitellogenin in other sea urchins. The protein in the gonads and coelomic fluids of both sexes appears to exist as a complex—as does MYP in unfertilized eggs—because in SDS-PAGE under nonreducing conditions bands occurred at around 270 kDa (Fig. 4f–j).

Extracts from testes at three maturational stages (Stages 1, 3, and 4) were subjected to SDS-PAGE and immunoblotting analyses (Fig. 5). As spermatogenesis progressed, the quantity of 170-kDa protein reactive with anti-MYP decreased in the testis. Mature testis (Stage 5) contained little of this protein (Fig. 5c, f).

Distribution of MYP in gonads during gametogenesis

Immunolocalization of MYP in the gonads is shown in Figure 6. The follicular lumina of immature testes and ovaries (Stage 1) were filled with nutritive phagocytes (Fig. 6A, G). The protein reactive with anti-MYP was stored in the nutritive phagocytes of both sexes (Fig. 6D, J). With the progress of gametogenesis, testes contained peripheral lines of spermatogonia and spermatocytes, and ovaries had a row of vitellogenic oocytes. The lumen of testes later filled with spermatozoa and that of ovaries with ripe ova (Fig. 6B, H). Spermatogenic cells and oocytes did not react with the anti-MYP, whereas ripe ova did (Fig. 6E, K). The nutritive phagocytes had degenerated but were still reactive with anti-MYP in Stage 3 gonads (Fig. 6E, K). In mature gonads (Stage 4), the gonadal lumina were filled with spermatozoa or ripe ova, and nutritive phagocytes were recognized only at the periphery of follicles (Fig. 6C, I). The protein disappeared

Figure 5. Five percent SDS-PAGE under reducing conditions and immunoblotting using anti-MYP of testicular extracts of *Pseudocentrotus depressus* at three maturational stages. (a, d) Stage 1; (b, e) Stage 3; (c, f) Stage 4. Amount of extract applied to each lane = 2 μl.

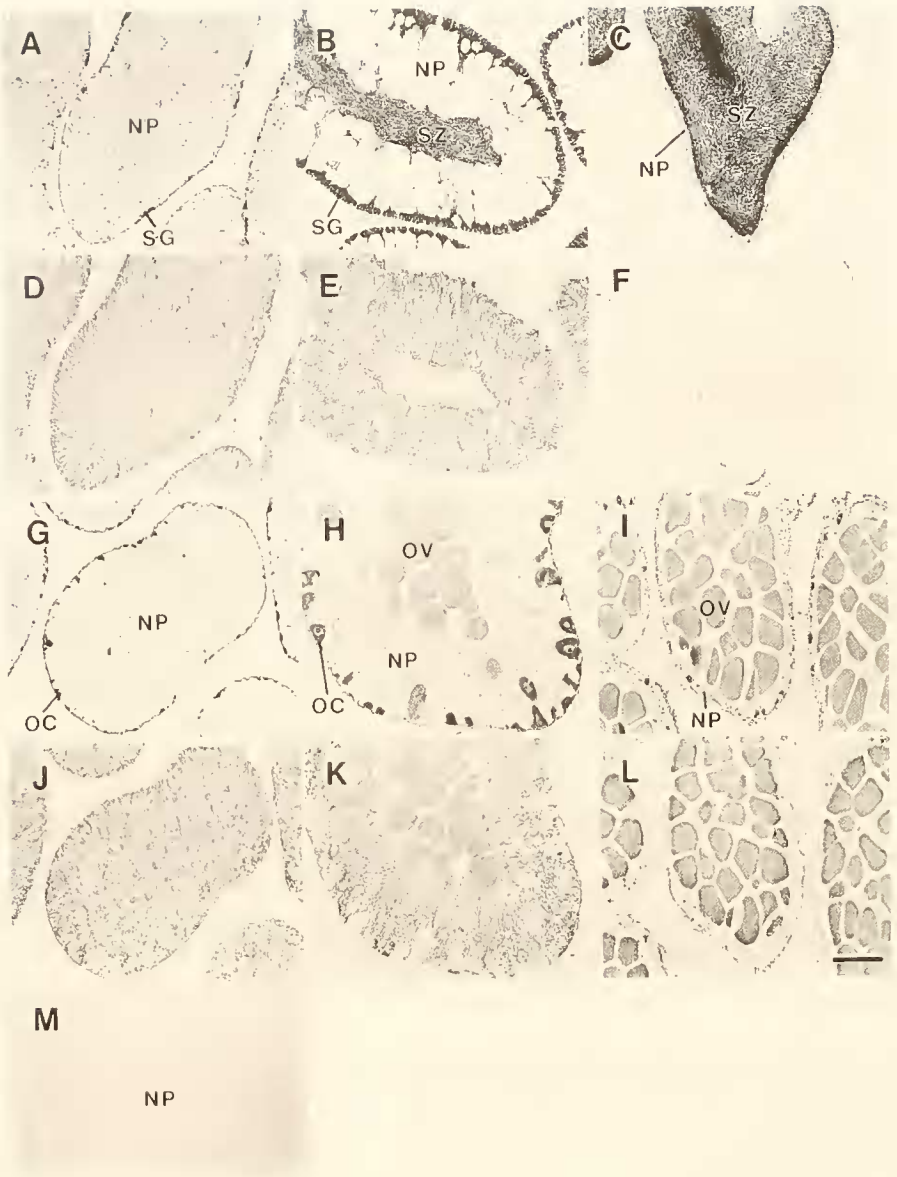

Figure 6. Immunolocalization of the protein reactive with anti-MYP in the testes (A–F and M) and ovaries (G–L) of *Pseudocentrotus depressus*. A–C and G–I were stained with hematoxylin and eosin; D–F and J–L were immunostained with anti-MYP; M was treated with normal rabbit serum instead of anti-MYP as an immunostaining control. (A, D, G, J, M) Stage 1: Nutritive phagocytes reacted with anti-MYP in both testis and ovary. (B, E, H, K) Stage 3: The spermatogenic cells and oocytes did not react with anti-MYP, but the ripe ova did react. Nutritive phagocytes were still reactive with anti-MYP. (C, F, I, L) Stage 4: The protein was not detected from the testis, but it was accumulated in the ripe ova in the ovary. NP, nutritive phagocyte; SG, spermatogonium and spermatocyte; SZ, spermatozoon; OC, oocyte; OV, ripe ovum. Scale bar = 100 μm.

from the testis (Fig. 6F), but in the ovary it accumulated in ripe ova as a yolk protein (Fig. 6L).

Discussion

This study demonstrates that prior to gametogenesis in the red sea urchin *P. depressus*, a protein identical to MYP is stored abundantly in the nutritive phagocytes of males as well as those of females. However, as gametogenesis proceeds, this protein decreases in quantity in the testis while it is accumulated in ripe ova as a yolk protein in the ovary. Histological observations have led to the suggestion that nutritive phagocytes in the testis are nutrient storage sites for spermatogenesis (Walker, 1982).

However, it is still unclear what kind of material functions as a nutrient storage in the testicular nutritive phagocytes. We propose that the MYP found in the testis functions as a nutrient for spermatogenesis in *P. depressus*. In oviparous animals, yolk protein is a nutrient source for embryogenesis and is usually found only in the female (Hara, 1987; Quinitio *et al.*, 1989, 1990; Suzuki *et al.*, 1992; Osada *et al.*, 1992). *P. depressus* is unique in apparently using a yolk protein as a nutrient for spermatogenesis.

Vitellogenin, a precursor of MYP, is abundant in the coelomic fluid of both males and females, and its role in the male sea urchin has been discussed (Harrington and Easton, 1982; Shyu *et al.*, 1986). Harrington and Easton (1982) postulated that vitellogenin performs an unknown physiological role required by both sexes, but related to the hermaphrodism observed in some echinoderms. Shyu *et al.* (1986) proposed that vitellogenin functions as an analog to the serum albumin of vertebrates, as a carrier protein, or as a store for amino acids. The possibility that male vitellogenin, like female vitellogenin, is a precursor of MYP has not been discussed because MYP storage in the testis had not been demonstrated. In female sea urchins, vitellogenin is incorporated into the nutritive phagocytes, then transported to the oocytes to be accumulated as MYP (Ozaki *et al.*, 1986; Harrington and Ozaki, 1986). We suggest that male vitellogenin is a precursor of MYP in the testis and is incorporated into the testicular nutritive phagocytes as a nutrient source for spermatogenesis. We think that the process until the incorporation into the gonad is probably the same in both sexes, although the final site for utilization is different.

MYP of *P. depressus* has a molecular weight of 170 kDa under reducing conditions, but vitellogenin has a slightly higher molecular weight (180 kDa). The same is true in *S. purpuratus:* the molecular weight of vitellogenin is 195 kDa, but that of MYP is 180 kDa (Harrington and Easton, 1982; Shyu *et al.*, 1986). Thus, a decrease in molecular weight from vitellogenins to MYPs seems to be a common phenomenon in sea urchins. It is probable that vitellogenin is slightly modified in molecular structure after its incorporation into the gonads. Shyu *et al.* (1986) presumed that this modification takes place in the oocytes, but we suggest that it occurs in the nutritive phagocytes of both sexes immediately after incorporation. We base our conclusion on the observation that the protein stored in the nutritive phagocytes is already modified to 170 kDa and little of the 180 kDa protein is detected in the gonads.

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